PROCESSING AND PRODUCTS
Research Note

Detection of *Salmonella enteritidis* in Incubated Pools of Egg Contents by Fluorescence Polarization and Lateral Flow Immunodiffusion

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ABSTRACT Efficient detection of *Salmonella enteritidis* inside eggs is critical for confirming that individual commercial laying flocks present a risk to public health. In most standard bacteriological culturing protocols, an initial incubation step is necessary to allow the typically very small population of *S. enteritidis* cells in pools of egg contents to multiply to more easily detectable levels. In the present study, two rapid methods were evaluated as alternatives to plating on selective media for detecting *S. enteritidis* in incubated egg pools. By using either fluorescence polarization or lateral flow immunodiffusion assays, *S. enteritidis* could be consistently detected in egg pools at 10^8 cfu/mL (and in most pools at 10^7 cfu/mL). Although the rapid assays were significantly less sensitive than culturing, they both were consistently able to detect contamination when pools of 10 eggs were inoculated with approximately 10 cfu of *S. enteritidis* and incubated for 72 h at 25°C.

(*Key words*: Salmonella enteritidis, egg, fluorescence polarization, lateral flow immunodiffusion)

INTRODUCTION

Eggs contaminated internally with *Salmonella enteritidis* are important sources of foodborne disease throughout the world (Angulo and Swerdlow, 1999; Centers for Disease Control, 2000). The colonization of reproductive tissues of laying hens by *S. enteritidis* is apparently responsible for bacterial deposition inside eggs (Okamura et al., 2001a,b). Detection of infected commercial laying flocks is thus a central objective of *S. enteritidis* control efforts (Hogue et al., 1997). A proposed national program in the United States would determine whether flocks are subject to regulatory action by a strategy of first screening for the presence of *S. enteritidis* in the laying house environment and then culturing eggs to detect internal contamination (President's Council on Food Safety, 1999).

Identification of the pathogen inside eggs provides incontrovertible evidence that flocks threaten public health. However, egg contamination occurs very infrequently (Ebel and Schlosser, 2000) and typically involves low levels of *S. enteritidis* cells (Humphrey et al., 1991; Gast and Holt, 2000). Accordingly, sensitive and consistent detection of *S. enteritidis* is possible only if large numbers of eggs are tested. The contents of 10 or more eggs are often pooled together to obtain a manageable total number of samples. Because this pooling introduces a further dilution of the already low *S. enteritidis* levels deposited in eggs, the pools are normally incubated for one or more days to allow bacterial multiplication to proceed sufficiently and allow detection by standard culture methods (Gast, 1993a,b). This initial pool incubation step is sometimes adequate to support bacterial recovery by plating onto agar media without intervening broth enrichment steps (Gast, 1993c; Gast and Holt, 1995, 1998). Alternatives to conventional plating, which would allow more rapid identification of *S. enteritidis* contamination in incubated egg pools, would further improve the efficiency of detection.

Fluorescence polarization technology is based on the inverse relationship between the sizes of molecules and their natural rates of spin in liquid media (Nielsen et al., 1996; Nasir and Jolley, 1999). The spin rates of labeled tracer molecules are reduced when they bind to specific antibody molecules. A beam of polarized light can be used to detect changes in the spin rates of molecules labeled with a fluorescent dye. This approach has already been successfully applied to detect antibodies to *S. enteritidis* in sera and egg yolks from experimentally infected hens (Gast et al., 2002a,b). The assay can also be adapted for antigen detection as a competitive assay in which

Abbreviation Key: FP = fluorescence polarization; LF = lateral flow immunodiffusion.

the binding of antibody to labeled tracer is reduced if the target antigen is also present in the test sample.

Lateral flow immunodiffusion technology (Bird et al., 1999) detects antigens using specific antibodies conjugated to colloidal gold particles. After samples diffuse into a reagent zone containing gold-conjugated antibodies, any antigen-antibody complexes that form travel through a membrane and are captured for visualization by additional antibodies to the target antigen. In the present study, both fluorescence polarization and lateral flow immunodiffusion assays were evaluated as alternatives to bacteriological culturing for detecting S. enteritidis in experimentally contaminated pools of egg contents.

MATERIALS AND METHODS

Detection of S. enteritidis Contamination at Known Concentrations in Egg Contents Pools

In each of two replicate trials, the entire liquid contents of eggs from our laboratory's specific-pathogen-free chicken flock were mixed by stomaching and used to prepare 40 pools of 10 mL each. A phage type 14b S. enteritidis isolate was grown overnight in tryptone soya broth.[2] Two serial 10-fold dilutions of this culture were prepared in 0.85% saline, and the concentrations of S. enteritidis cells in these dilutions were confirmed by subsequent plate counts. Three groups of 10 egg contents pools were each contaminated with 1 mL of either the original S. enteritidis culture or the two dilutions, yielding final concentrations of 1.0×10^8 cfu/mL, 1.0×10^7 cfu/mL, and 1.0×10^6 cfu/mL. A fourth group of 10 egg pools was not contaminated. Each egg contents sample was then mixed by stomaching and tested to detect S. enteritidis contamination by three methods.

For bacteriological culturing, 1 mL from each egg pool was transferred to 9 mL of Rappaport-Vassiliadis broth[2] and incubated for 24 h at 37°C. A loopful from each culture was then streaked onto plates of brilliant green agar[3] and again incubated for 24 h at 37°C. The identity of suspected S. enteritidis colonies was confirmed biochemically and serologically (Waltman et al., 1998). For the Reveal lateral flow immunodiffusion test[4] (LF) and the fluorescence polarization immunoassay[5] (FP), 2 mL from each egg pool was diluted in 8 mL PBS and mixed by vortexing. The samples were immersed in a boiling water bath for 15 min, and the upper aqueous phase was removed for testing.

The LF test (Bird et al., 1999) was performed by adding 120 μL of diluted sample to the test device and incubating

at room temperature for 15 min. The development of lines in the test window indicated the capture of complexes of S. enteritidis cells, antibodies to S. enteritidis flagella, and a colloidal gold indicator. The FP test (Nielsen et al., 1996; Gast et al., 2002a,b) was performed by adding 100 μL of diluted sample to 1 mL of PBS containing polyvalent antiserum to S. enteritidis O-polysaccharide at a 1:300 dilution and determining a baseline intensity value in a Sentry reader.[5] After this blanking step, 10 μL of tracer[5] (a fluorescein-labeled preparation of S. enteritidis O-polysaccharide purified using a polymyxin B column) was added to the sample, and the FP reading was taken after 2 min. The presence of S. enteritidis in the sample reduced the binding of tracer by antibodies and thereby also reduced the FP reading. Samples were considered positive if the FP value was lower than the mean FP value for the uncontaminated negative control samples by more than two standard deviations.

Detection of S. enteritidis After Incubation of Contaminated Egg Contents Pools

In each of two replicate trials, the contents of 130 eggs from our laboratory's specific-pathogen-free chicken flock were used to prepare 13 pools of 10 eggs each. Five pools were supplemented with 40 mL of 60 mg/mL ferrous sulfate,[6] five pools were supplemented with 40 mL of tryptone soya broth prepared at five times the usual concentration, and three pools were supplemented with 20 mL of each additive. These additives have been shown to improve the growth rate of S. enteritidis in incubating egg contents pools (Gast et al., 1998). A phage type 14b isolate of S. enteritidis was prepared as described above and diluted in 0.85% saline to approximately 10 cfu/mL. Each of the 10 egg pools containing a single additive was contaminated with 1 mL of S. enteritidis culture. The three pools containing both additives were retained as negative controls. All pools were mixed by stomaching for 30 s and incubated at 25°C for 72 h. The final numbers of S. enteritidis cells reached in each sample were determined by spreading dilutions onto brilliant green agar and counting characteristic colonies after 24 h of incubation at 37°C. Two milliliters of each incubated egg pool was also transferred to 8 mL of PBS, boiled for 15 min, and tested by the LF and FP assays as described above.

Statistical Analysis

For each replicate trial, significant differences ($P < 0.05$) between testing methods in the mean frequency of detection of S. enteritidis in experimentally contaminated egg contents pools were determined by Kruskal-Wallis analysis of variance followed by Dunn's multiple comparison test. Data were analyzed using Instat biostatistics software.[7] Because the statistical relationships between treatment groups were similar for the two trials, the results were combined for presentation.

TABLE 1. Detection of *Salmonella enteritidis* in pools of egg contents by fluorescence polarization, lateral flow immunodiffusion, and bacteriological culturing

Detection method	Pools contaminated with known levels of *S. enteritidis* (cfu/mL)[1]			Pools contaminated initially with 10 cfu of *S. enteritidis* and then incubated[2]
	10^6	10^7	10^8	
	(S. enteritidis detected/total)			
Fluorescence polarization	0/20	18/20	20/20	20/20
Lateral flow immunodiffusion	0/20	18/20	20/20	20/20
Bacteriological culturing	20/20	20/20	20/20	20/20

[1]Pools of the contents of 10 eggs were contaminated with specific concentrations of *S. enteritidis* and tested immediately by each of the three detection methods.

[2]Pools of the contents of 10 eggs were contaminated with 10 cfu of *S. enteritidis*, incubated for 72 h at 25°C, and then tested by each of the three detection methods.

RESULTS AND DISCUSSION

When pools of egg contents were contaminated with 10^8 cfu/mL of *S. enteritidis*, all of the samples were detected as positive by culturing, FP, and LF (Table 1). At a contamination level of 10^7 cfu/mL in egg pools, *S. enteritidis* was detected in all of the samples by culturing and in 90.0% of the samples by either FP or LF. However, although all egg pools inoculated with 10^6 cfu/mL of *S. enteritidis* were detected as positive by culturing, neither FP nor LF was able to detect contamination at this level. No test gave positive results with uncontaminated egg pools. No differences between test methods were evident at the 10^8 or 10^7 cfu/mL contamination levels ($P > 0.05$), but culturing was more sensitive ($P < 0.0001$) than either of the other methods at the 10^6 cfu/mL level.

When pools of the contents of 10 eggs were contaminated with an initial dose of approximately 10 cfu of *S. enteritidis* and then incubated for 72 h at 25°C, bacteriological culturing indicated that all pools supplemented with either ferrous sulfate or concentrated tryptone soya broth reached final levels of at least 1.0×10^8 cfu/mL of *S. enteritidis*. The FP and LF tests both detected *S. enteritidis* in all of these egg pools after incubation (Table 1).

Both rapid methods examined in the present study were consistently able to detect *S. enteritidis* contamination in egg pools at a level of 10^8 cfu/mL (and were usually effective in doing so at 10^7 cfu/mL). Because most available evidence suggests that eggs rarely contain more than a few hundred *S. enteritidis* cells (Humphrey et al., 1991; Gast and Holt, 2000), a substantial incubation period is apparently necessary before the FP and LF tests can dependably identify contaminated egg pools. Incubating egg pools for 72 h at 25°C (or for 24 h at 37°C) has been shown to support the detection of small numbers of *S. enteritidis* by a direct plating culture approach, which has a sensitivity of 10^5 cfu/mL (Gast 1993a,c; Gast and Holt, 1995). The present study indicates that the same egg pool incubation strategy should be adequate to allow *S. enteritidis* detection by FP or LF.

The previously reported sensitivities of ELISA (Price et al., 1995) and PCR (Burkhalter et al., 1995; McElroy et al., 1996) techniques are similarly unlikely to permit effective detection of *S. enteritidis* in eggs without an extended initial pool incubation step. Iron or concentrated broth media supplementation to increase the growth rate of *S. enteritidis* in egg pools has been helpful for improving the efficiency of detection by direct plating (Gast and Holt 1995, 1998; Chen et al., 2001). Recent experiments have shown that incubation for less than 72 h at 25°C (or 24 h at 37°C) can be sufficient to allow the multiplication of *S. enteritidis* to detectable levels in supplemented egg pools (R. Gast, unpublished data). Centrifugation (McElroy et al., 1995) or immunomagnetic separation (Cudjoe et al., 1994; Holt et al., 1995) can be used to concentrate *S. enteritidis* contaminants from incubated egg pools. Nevertheless, as the current experiments illustrate, multiple-step broth enrichment culture methods generally provide considerably more sensitive detection of *S. enteritidis* in egg pools than can be obtained via any of the more rapid methods (Gast, 1993c).

Culturing incubated egg pools by direct plating generally requires at least 48 h to obtain confirmed results. Both FP and LF are extremely simple and rapid, providing results within 15 min. To constitute viable alternatives to direct plating, rapid tests would have to demonstrate comparable sensitivity of detection at a reasonable cost. The specificity at which *S. enteritidis* can be identified using these assays would also need to be established, as other *Salmonella* serotypes are sometimes found inside eggs (Ebel et al., 1993). In the present study, neither rapid assay could match the previously documented performance of culturing by either direct plating or broth enrichment. When protecting public health is at issue, speed is arguably a less important test attribute than detection sensitivity.

ACKNOWLEDGMENT

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